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The protective effects of melatonin against cryopreservation-induced oxidative stress in human sperm

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Abstract

Reactive oxygen species (ROS) production and lipid peroxidation during cryopreservation harm sperm membrane and as a result reduce the recovery of motile sperm. The antioxidant effects of melatonin on different cells have been widely reported. This study was aimed to evaluate changes in post-thaw motility, viability, and intracellular ROS and malondialdehyde (MDA) in response to the addition of melatonin to human sperm freezing extender. Semen of 43 fertile men was collected and each sample was divided into eight equal aliquots. An aliquot was analyzed freshly for viability, motility, and intracellular ROS and MDA. Melatonin was added to the recommended human freezing extender to yield six different final concentrations: 0.001, 0.005, 0.01, 0.05, 0.1, and 1 mM. A control group without melatonin was also included. Two weeks after cryopreservation, samples were thawed and pre-freeze analyses repeated. Obtained results showed that cryopreservation significantly ($P < 0.05$) reduces viability and motility, but increases intracellular ROS and MDA of human sperm. The semen extender supplemented with various doses of melatonin (except for 0.001 mM) significantly ($P < 0.05$) increased motility and viability, but decreased intracellular ROS and MDA levels of cryopreserved sperm after the thawing process, as compared with the control group. We also found that the most effective concentration of melatonin in protecting human spermatozoa from cryopreservation injuries was 0.01 mM. These findings suggest that melatonin exerts its cryoprotective effects on spermatozoa possibly by counteracting intracellular ROS, and thereby reduces MDA generation. This finally leads to increase of post-thaw viability and motility of cryopreserved spermatozoa.

Keywords

cryopreservation, malondialdehyde, melatonin, motility, oxygen reactive species

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Cryopreservation includes exposure of gametes to anti-freezing substances, cooling to subzero temperatures, storing, melting, and ultimately, eliminating the anti-freezing substance, when used, with a return to a physiological environment that will allow subsequent development. Sperm cryopreservation has been vastly used in sperm banking prior to chemotherapy or radiation treatment for cancer, donor sperm for couples without a male partner, and various etiologies of male factor infertility.[1] Cryopreservation can prevent repeating surgical procedures used as cures for azoospermic conditions like microsurgical epididymal sperm aspiration (MESA) and testicular sperm

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extraction (TESE). In addition, frozen semen permits precise examination of the existence of infections such as HIV and HBS.[2]

Bunge and Sherman presented the first pregnancy resulting from the thawing of cryopreserved sperm in 1953.[3] In spite of the considerable progress made in the sperm cryopreservation field, it is accompanied by some shortcomings. For example, the motility and viability of sperm decrease significantly after the freezing and thawing process. This is in part due to the fact that cryopreservation induces oxidative stress and, as a result, the formation of reactive oxygen species (ROS). The polyunsaturated fatty acids of the sperm plasma membrane are susceptible to ROS damage inasmuch as its cytoplasm contains low concentrations of free radical scavenging enzymes.[4] ROS start a chain of chemical reactions called lipid peroxidation, which generates reactive metabolites, such as malondialdehyde (MDA). Lipid peroxidation reduces membrane fluidity and the activity of membrane enzymes and ion channels, resulting in the inhibition of normal cellular mechanisms required for sperm motility and fertility.[5] A suitable approach for the enhancement of cryopreserved sperm is adding different substances such as antioxidants to the freezing solutions.[6] Antioxidants seem to be of great clinical importance by reducing the amount of free radicals and oxidative stress and, as a result, improving fertility potential in assisted reproductive technology (ART) techniques.[7]

Melatonin, thought to be the major output hormone of the pineal gland, plays important roles in the regulating of some physiological events. This molecule has the capability of removing and neutralizing free radicals such as hydroxyl, peroxyl, and peroxynitrite anions. In humans, it has been observed that melatonin relieves the sperm mitochondrial oxidative stress caused by ROS.[8]

In spite of the massive effort, due to the limitation in human resources like collecting and preserving samples, many aspects of the effect of antioxidants on maintaining the sperm parameters after freezing has remained vague and to some extent, unknown. For this reason, this study focused on examining the effect of melatonin antioxidant potential on the oxidative stress and human sperm parameters after freezing.

Materials and methods

Sample preparation

Semen samples were collected from 43 fertile men with sperm density more than 20 million per mL after 3–4 days of sexual abstinence in sterile plastic containers by masturbation in the privacy room adjacent to the laboratory. Ethical approval was obtained from the Ethics Committee of Ilam University of Medical Sciences. Informed consent was obtained from all donors. All subjects included in the study had no abnormalities during examination. To liquefy the clotted semen, samples were kept in a 37°C incubator for 20–30 min. All samples were analyzed by a computer-assisted sperm analysis (CASA) system to quantify sperm count, motility parameters, and morphometric features. According to the World Health Organization (WHO) criteria,[9] sperm motility was divided into four classes: (1) class A or grade IV: Sperm with straight, progressive, and fast motility; (2) class B or grade III: Sperm with non-linear progressive, and slow motility; (3) class C or grade II: Sperm with non-progressive or rotational motility; and (4) class D or grade I: Sperm with any motility. After washing with washing medium (SAGE, USA), centrifuge (Hettich, 5000 g) was applied to separate the semen and sperm. The newly formed cell plaque was rewashed with the sperm washing medium and subsequently mixed with the cryopreservation solution (CS) drop by drop and slowly. Each sample was subdivided into eight equal parts: a part was examined fresh without being exposed to CS, another washed in CS devoid of melatonin (control group). Concentrations 0.001, 0.005, 0.01, 0.05, 0.1, and 1 mM of melatonin were added into six remaining parts for freezing. The melatonin-treated samples and control group were kept for 10 min at room temperature, 10 min in –20°C, 10 min in –70°C and finally transferred to a liquid nitrogen tank. After 2 weeks, samples were taken out and thawed. This process consisted of keeping the newly extracted cryotubes at room temperature for 1–2 min and afterwards in water bath in 37°C for 5 min. Samples were centrifuged at 300 x g, the supernatant was removed, and after adding sperm washing solution, the pre-freeze analysis was repeated.

ROS assay

The determination of ROS was carried out using an ELISA kit (BlueGene Biotech Co. Ltd., PR China)

Table 1. Fresh semen analysis of the study subjects before cryopreservation.

Characteristics		Mean ± SD
Age (years)		32.51 ± 4.13
Volume (mL)		4.38 ± 1.25
pH		7.55 ± 0.22
Viability (%)		82 ± 12.97
Sperm concentration (10^6/mL)		65.50 ± 22.90
Sperm with normal morphology (%)		50.73 ± 14.66
Sperm motility (%)	Class A	43 ± 10.38
	Class B	20 ± 1.64
	Class C	12 ± 1.51
	Class D	25 ± 4.20
Sperm ROS (μg/10^6 sperm)		0.12 ± 0.02
Sperm MDA (pmol/10^6 sperm)		73 ± 11

according to its manual instruction. In brief, sperm samples were washed three times in PBS. Afterwards, samples were resuspended in PBS and subjected to ultra-sonication three times. The sample was centrifuged at 1000 x g for 15 min at 2–8°C to remove cellular debris, and supernatant was used for the assay. Finally, ROS assay was performed immediately. By plotting a standard curve, the concentration of ROS in each sample was determined. The results were expressed as μg of ROS/10^6 sperm.

MDA assay

The level of sperm MDA was determined through the thiobarbituric acid (TBA) reaction according to the method described by Rao et al.[10] The MDA determination was carried out as follows: 250 μL of sperm lysate (as described above) sample, 50 μL of Butilate Hidroxitoluene (BHT, 50 mmol/L), and 750 μL of trichloric acetic acid (TCA 10 %) were incubated for 10 min at 4°C and then centrifuged for 10 min at 1000 rpm. In the next step, 750 μL of the supernatant were mixed with 750 μL of thiobar-bituric acid 5%. This mixture was heated for 15 min at 100°C, cooled and read at 532 nm. By plotting a standard curve, the concentration of MDA in each sample was determined. Values were expressed as pmol of MDA/10^6 sperm.

Statistical analysis

All data were expressed as the mean ± Standard Deviation (SD) of three independent experiments. Statistical analyses were performed using SPSS 19 (SPSS, Chicago, IL, USA). Comparisons among groups were performed with the one-way analysis of variance (ANOVA) test. If statistical significance was found, the Tukey's post-hoc test was performed. Values of $P < 0.05$ were considered statistically significant.

Results

Sperm viability, motility, and ROS and lipid peroxidation (using MDA assay) levels of participants before cryopreservation are shown in Table 1.

Figure 1 shows the viability (%) in different groups of sperm after cryopreservation. The viability significantly decreased in all melatonin-treated and untreated (control) sperm groups in comparison with the fresh sperms ($P < 0.05$) after cryopreservation (Table 1 and Figure 1). However, the viability of sperm groups treated with the varying concentrations of melatonin significantly ($P < 0.05$) increased (except for 0.001 mM melatonin) with respect to the control group. After cryopreservation, the highest viability was observed for sperm group treated with 0.01 mM melatonin, which was about 1.6-fold of the control group.

Comparing Table 1 and Figure 2 indicated that the total motility significantly decreased in all melatonin-treated and control groups in comparison with the fresh sperm ($P < 0.05$) after cryopreservation. However, the motility of sperm groups treated with the varying concentrations of melatonin significantly ($P < 0.05$) increased (except for 0.001 mM melatonin) with respect to the control group. After cryopreservation, the highest motility was observed for the sperm group treated with 0.01 mM melatonin, which was about 1.75-fold of the control group.

To detect whether melatonin could reduce cryopreservation-induced ROS production, we determined ROS concentration in different groups of sperm which were treated with varying doses of melatonin. Comparing Table 1 and Figure 3 indicated that the ROS concentration significantly decreased in all melatonin-treated and control groups in comparison with the fresh sperm ($P < 0.05$) after cryopreservation. However, the ROS concentration in sperm groups treated with the varying doses of melatonin significantly ($P < 0.05$) increased (except for 0.001 mM melatonin) with respect to the control group. After cryopreservation, the lowest ROS concentration was observed

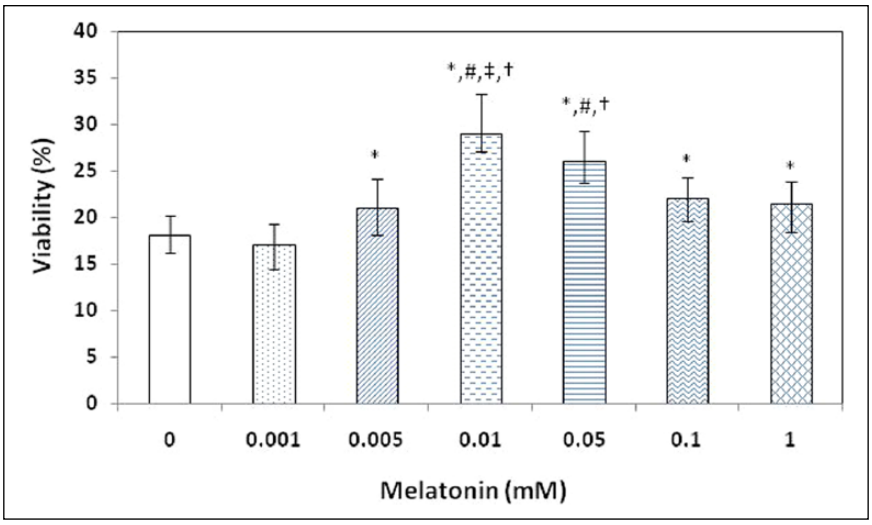

Figure 1. The effect of varying concentrations of melatonin on sperm viability (%) after cryopreservation. Data are expressed as mean ± SD of five different experiments. *P <0.05 vs. control and 0.001 mM melatonin-treated groups. #P <0.05 vs. 0.005 mM melatonin-treated group. ‡P <0.05 vs. 0.05 mM melatonin-treated group. †P <0.05 vs. 0.1 and 1 mM melatonin-treated groups.

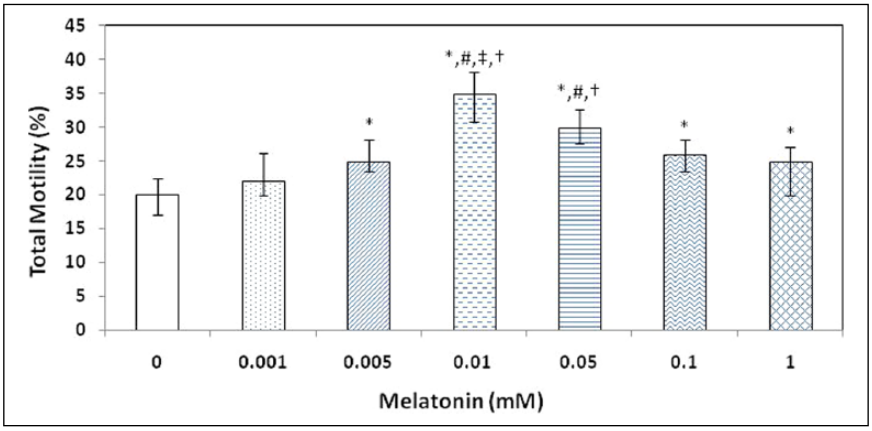

Figure 2. The effect of varying concentrations of melatonin on sperm's total motility (%) after cryopreservation. Data are expressed as mean ± SD of five different experiments. *P <0.05 vs. control and 0.001 mM melatonin-treated groups. #P <0.05 vs. 0.005 mM melatonin-treated group. ‡P <0.05 vs. 0.05 mM melatonin-treated group. †P <0.05 vs. 0.1 and 1 mM melatonin-treated groups.

in the sperm group treated with 0.01 mM melatonin, which was about 47% of the control group. The ROS concentrations in 0.005, 0.05, 0.1, and 1 mM melatonin-treated sperm groups reduced to 79%, 59%, 66%, and 68% of the control group, respectively.

Lipid peroxides are derived from the peroxidation of polyunsaturated fatty acids, and the most abundant lipid peroxide is MDA. Therefore, the content of sperm lipid peroxidation was determined by measuring MDA.[10] To investigate whether melatonin could reduce cryopreservation-induced MDA production, we measured MDA concentration in melatonin-treated sperm groups. Comparing Table 1 and Figure 4 indicated that the MDA concentrations significantly increased in all melatonin-treated and control groups after cryopreservation, as compared with the fresh sperms (P <0.05). However, the MDA concentration in sperm groups treated with the varying doses of melatonin significantly (P <0.05) decreased (except for 0.001 mM melatonin) when compared with the control group. After cryopreservation, the lowest MDA concentration was observed in the sperm group treated with 0.01 mM melatonin, which was about 63% of the control group. The MDA concentrations in 0.005, 0.05, 0.1, and 1 mM melatonin-treated sperm groups reduced to

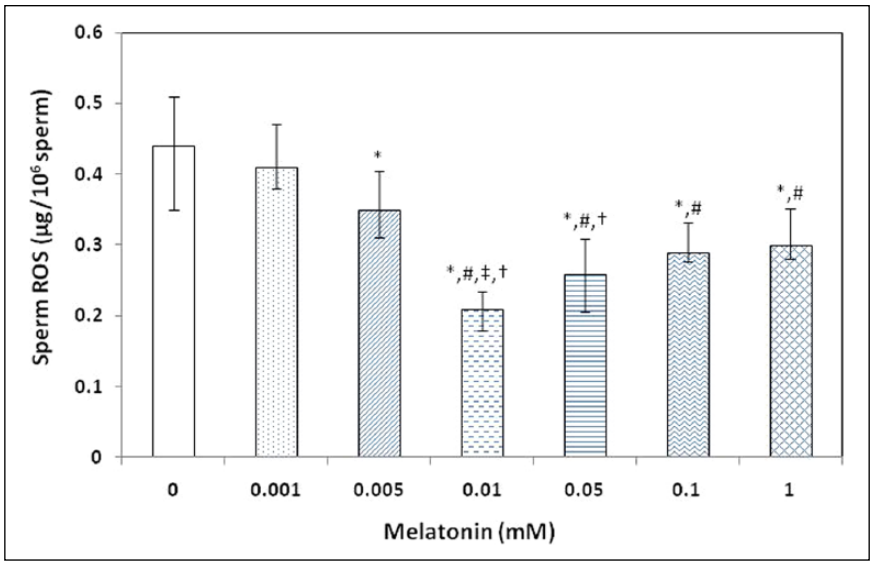

Figure 3. The effect of varying concentrations of melatonin on sperm's ROS concentration (µg/10^6 sperm) after cryopreservation. Data are expressed as mean ± SD of five different experiments. *P <0.05 vs. control and 0.001 mM melatonin-treated groups. #P <0.05 vs. 0.005 mM melatonin-treated group. ‡P <0.05 vs. 0.05 mM melatonin-treated group. †P <0.05 vs. 0.1 and 1 mM melatonin-treated groups.

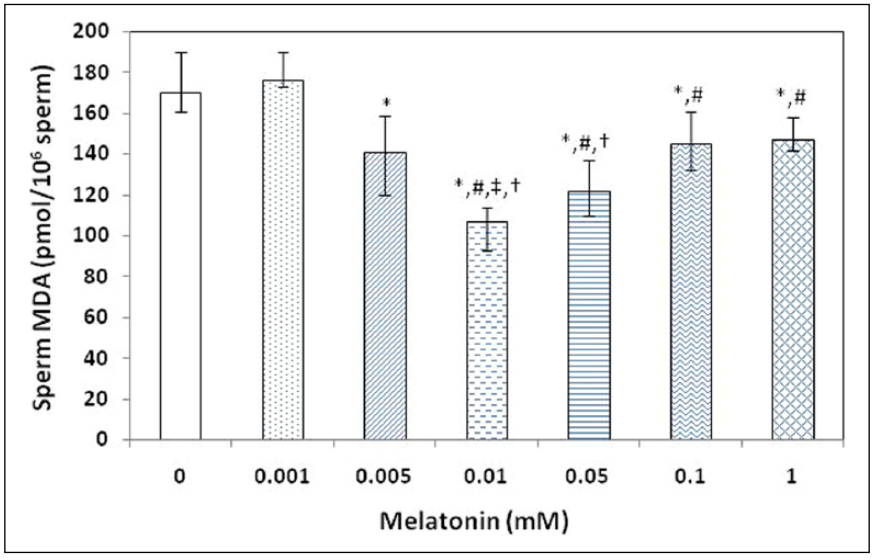

Figure 4. The effect of varying concentrations of melatonin on sperm's MDA concentration (pmol/10^6 sperm) after cryopreservation. Data are expressed as mean ± SD of five different experiments. *P <0.05 vs. control and 0.001 mM melatonin-treated groups. #P <0.05 vs. 0.005 mM melatonin-treated group. ‡P <0.05 vs. 0.05 mM melatonin-treated group. †P <0.05 vs. 0.1 and 1 mM melatonin-treated groups.

82%, 71%, 85%, and 86.5% of the control group, respectively.

Discussion

Cryopreservation can damage spermatozoa in different levels structures, such as acrosome membranes or mitochondria, and finally reduce its fertilizing ability.[11,12] Furthermore, a significant reduction in the level of spermatozoa antioxidants has been reported as one of the causes of the enhanced susceptibility of these cells to peroxidative injuries after cryopreservation.[13] For these reasons, protective effects of different exogenous antioxidants on sperm quality and function after thawing has been explored.[7,13–15] In recent years,

reports about the beneficial effects of melatonin in protecting spermatozoa from different kinds of injury have emerged. Several studies reported that melatonin protected animal spermatozoa from adverse effects of peroxidative agents.[16,17] Melatonin directly neutralizes a high number of toxic free radicals.[18] However, the possible role of melatonin in protecting spermatozoa against oxidative stress has not been fully identified up to now. To our knowledge, this is the first report exploring the protective effect of several concentrations of melatonin during human semen cryopreservation. Our results showed that the addition of 0.01 mM melatonin to the human semen freezing medium improves significantly spermatozoa viability, motility, intracellular ROS and MDA concentrations post thawing, and thereby fertilizing ability.

Our results generally showed that the addition of melatonin to the freezing media increases viability and motility of cryopreserved sperms. The efficient concentration of melatonin that maintained highest viability and motility after cryopreservation was observed to be 0.01 mM. To our knowledge, there is no report about the effect of melatonin on the viability and motility of cryopreserved human sperm. However, studies on ram and bull sperm demonstrated that semen cryopreservation with 2 mM melatonin increases viability and motility of post-thawed sperm.[18,19]

One of the most important reasons for reduced sperm survival after the freezing-thawing process seems to be damage to the membrane system through lipid oxidation from increased levels of ROS.[20] Several studies have revealed that melatonin can improve sperm function, possibly by scavenging of ROS, but the effect of melatonin on the ROS concentration of human sperm after cryopreservation has not previously been assessed.[8,21,22] In accordance with several previous studies, the results of the present study showed that ROS concentration significantly increased in post-thawed sperm when compared with fresh sperm.[20,23,24] It has been shown that the protective effect of melatonin on cryopreservation injuries occurs in a dose-dependent manner.[18,25,26]) Therefore, in the present study, different concentrations of melatonin (0.001–1 mM) in the freezing medium were assayed. We observed that the addition of melatonin (0.005–1 mM) to freezing media significantly reduces ROS generation after cryopreservation, and the lowest ROS concentration was observed in sperm group treated with 0.01 mM melatonin. To our knowledge, this finding has not previously been reported.

In the present study, MDA concentration, which is an index of ROS-induced lipid peroxidation, was measured in human sperm before and after cryopreservation and in the presence of several doses of melatonin. Our results indicated that MDA concentration significantly increased (nearly 2.5-fold) in post-thawed sperms when compared with fresh sperm. This finding was in agreement with the findings of several previous studies.[4,27,28]

We also found that the MDA concentration significantly decreased in sperm groups treated with 0.005, 0.01, 0.05, 0.1, and 1 mM of melatonin when compared with the control group. The lowest MDA concentration was observed in the sperm group treated with 0.01 mM melatonin, which was about 63% of the control group. Despite an extensive literature search, we did not find any related study that shows antioxidant effects of melatonin against cryopreservation-induced MDA generation in human sperm. However, a study by Ashrafi et al. showed that supplementation of the freezing media with 3 mM melatonin decreased MDA level in Holstein bulls.[19]

In addition, in their study, Gavella et al. showed that human sperm incubated in the presence of melatonin 6 mM exhibited a significant decrease in the rate of Iron/ascorbate-induced MDA generation.[8] In addition, in a study conducted by Perumal et al. in 2013, it was found that a sperm group treated with 3 mM of melatonin had significantly increased motility and viability in comparison with those treated with 1, 2, and 4 mM melatonin and the control group.[29] It has been demonstrated that melatonin, via its ROS scavenging activity, significantly stimulated and supported fertilization and early development of mouse embryo at concentration 10^{-6} to 10^{-4} M. It has also been reported by El-Raey that 0.1 mM of melatonin significantly decreased the rate of lipid peroxidation. On the other hand, it significantly enhanced post-thawing viability, motility, glutathione reductase and superoxide dismutase activity, and finally total antioxidant capacity.[30] The differences between the results of these studies and our study may be related to the difference in source of sperm and methodology.

In conclusion, the addition of melatonin to human semen freezing extender can protect spermatozoa

from the adverse effects of cryopreservation, as evidenced by post-thaw viability, motility, intracellular ROS and MDA concentrations, with 0.01 mM being the most effective concentration. These findings suggest that melatonin exerts its cryoprotective effects on spermatozoa possibly by counteracting intracellular ROS, and thereby reduces MDA generation. This finally leads to an increase in post-thaw viability and motility of cryopreserved spermatozoa.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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